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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c)

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TITLE OF THE INVENTION (500 characters max)

USE OF ALKALINE PHOSPHATASE FOR THE DETOXIFICATION OF LPS PRESENT AT MUCOSAL BARRIERS

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Respectfully submitted,

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Initial 2/4/04

Use of Alkaline Phosphatase for the detoxification of LPS present at mucosal barriers

Field of the invention

The current invention relates to the field of medicine and in particular to the use of LPS detoxifying and neutralizing enzymes. The present invention also relates to the field of pharmacy and in particular to the pharmaceutical use of alkaline phosphatase enzymes.

Background of the invention

Lipopolysaccharides (LPS; also referred to as endotoxins) are present in the cell walls of Gram-negative bacteria. When LPS is presented to a vertebrate body it stimulates the innate and cellular immune responses in a wide variety of cell types. The production of cytokines and chemokines (such as TNF's, various interleukines, interferons and others) will attract and activate cells of the immune system, which may culminate ultimately in an LPS induced systemic inflammatory response syndrome (SIRS) under certain conditions.

LPS or endotoxins are toxic to most mammals and regardless of the bacterial source, all endotoxins produce the same range of biological effects in the animal host. The injection of living or killed Gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of non-specific pathophysiological reactions such as: fever, tachycardia, tachypneu, hyper or hypothermia, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, organ dysfunction and may even result in shock and death.

Injection of small doses of endotoxin results in a proinflammatory response in most mammals, but the dose response range and steepness thereof varies significantly with the species and even within species may differ significantly depending on e.g. LPS-tolerance. The sequence of pro-inflammatory events follows a regular pattern (inflammatory cascade): (1) latent period; (2) physiological distress (diarrhea, prostration, shock); and in case of severe septic shock and multiple organ failure may result in (3) death. How soon death occurs varies on the dose of the endotoxin, route of administration, and species of animal.

The physiological effects of endotoxin are mainly mediated by the lipid A-moiety of LPS. Since Lipid A is embedded in the outer membrane of bacterial cells, it only exerts its toxic effects when released from multiplying cells in a soluble form, or when the bacteria are lysed as a result of autolysis, complement and the membrane attack complex (MAC), ingestion and killing by phagocytes, or killing with certain types of antibiotics. LPS released into the bloodstream can be neutralised by many blood components to a certain degree, amongst which several plasma lipids and proteins, among which LPS-binding proteins. The LPS-binding protein complex interacts with CD14 and Toll like receptors on monocytes and macrophages and through other receptors on endothelial cells. In monocytes and macrophages three types of events are triggered during their interaction with LPS:

Firstly, production of cytokines, including IL-1, IL-6, IL-8,, tumor necrosis factor (TNF) and platelet-activating factor. These in turn stimulate production of prostaglandins and leukotrienes. These are powerful mediators of inflammation and septic shock that accompanies endotoxin toxemia. LPS activates macrophages to enhanced phagocytosis and cytotoxicity. Macrophages are stimulated to produce and release lysosomal enzymes, IL-1 ("endogenous pyrogen"), and tumor necrosis factor (TNFalpha), as well as other cytokines and mediators.

Secondly, activation of the complement cascade. C3a and C5a cause histamine release (leading to vasodilation) and effect neutrophil chemotaxis and accumulation. The result is inflammation.

Finally, activation of the coagulation cascade. Initial activation of Hageman factor (blood-clotting Factor XII) can activate several humoral systems resulting in coagulation: a blood clotting cascade that leads to coagulation, thrombosis, acute disseminated intravascular coagulation, which depletes platelets and various clotting factors resulting in internal bleeding and also activation of the complement alternative pathway (as above, which leads to inflammation). Plasmin is activated which leads to fibrinolysis and hemorrhaging and kinin activation releases bradykinins and other vasoactive peptides which causes hypotension. The net effect is induction of inflammation, intravascular coagulation, hemorrhage and shock.

LPS also acts as a B cell mitogen stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins, especially IgG and IgM.

The physiological activities of LPS are mediated mainly by the Lipid A component of LPS. Lipid A is a powerful biological response modifier that can stimulate the mammalian immune system. During infectious disease caused by Gram-negative bacteria, endotoxins released from, or part of, multiplying cells have similar effects on animals and significantly contribute to the symptoms and pathology of the disease encountered. The primary structure of Lipid A has been elucidated and Lipid A has been chemically synthesized. Its biological activity appears to depend on a peculiar conformation that is determined by the glucosamine disaccharide, the PO_4 groups, the acyl chains, and also the KDO-containing inner core of the LPS molecule.

Alkaline phosphatase (AP), has been described earlier as a key enzyme in the dephosphorylation of LPS (endotoxin) under physiological conditions both in vitro and in vivo as a natural response to detoxify and neutralise LPS (US patent 6,290,952, Poelstra et al., Am J Pathol. 1997 Oct;151(4):1163-9).

Reports on the enzyme activity of AP involve its extremely high pH optimum for the usual exogenous substrates and its localization as an ecto-enzyme. Endotoxins are molecules that contain several phosphate groups and are usually present in the extracellular space. AP is able to dephosphorylate this bacterial product at physiological pH levels, by removing phosphate groups from amongst others the toxic lipid A moiety of LPS. As phosphate residues in the lipid A moiety determine the toxicity of the molecule, the effect of the AP inhibitor levamisole in vivo using a septicemia model in the rat confirmed the specificity of AP for LPS containing phosphate groups (Poelstra et al., 1997). The results demonstrated that inhibition of endogenous AP by levamisole significantly reduces survival of rats intraperitoneally injected with E. coli bacteria, whereas this drug does not influence survival of rats receiving a sublethal dose of the gram-positive bacteria Staphylococcus aureus, illustrating a crucial role for this enzyme in host defense. The effects of levamisole during gram-negative bacterial infections and the localization of AP as an ecto-enzyme in most organs as well as the induction of enzyme activity during inflammatory reactions and cholestasis is in accordance with such a protective role.

The prime source of LPS exposure in the human body are the gram negative microorganisms that live within the human digestive or gastrointestinal (GI) tract. There are far more bacteria in the digestive system than there are on the skin or other parts of the body, making the GI tract and GI mucosa the main route of entry for LPS

into the circulation. An average adult carries about 100 trillion bacteria in the intestines, most of which locate in the colon, contributing to 1-1.5kg of his body weight. There are more than 400 species of bacteria found in the digestive system. These include both beneficial (commensal) and potentially harmful (pathogenic) species, which continually compete to maintain a well-balanced intestinal flora.

Mucosal surfaces, and in particular (but not limited to) the intestinal mucosa, are exposed to this wide variety of commensal and potentially pathogenic bacteria, among which many gram negative endotoxin / LPS producing, Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, *Helicobacter*, *Chlamydia* and other leading pathogens. The intestinal epithelium is of particular importance as it forms a dynamic barrier that regulates absorption of nutrients and water and at the same time restricts uptake of microbes and other noxious materials such as LPS from the gut lumen.

It is well established that a major fraction of LPS influx from the lumen of the gut through the mucosal lining into the circulation of a vertebrate body is mediated through chylomicrons (Harris et al., 1998, 2000, 2002). Coincidental with ingestion of lipids and chylomicron introduction in circulation, capable of carrying LPS, a significant increase in lymphatic AP derived from the GI-tract is reported (Nauli et al., 2002). LPS-influx through the GI-barrier is increased normally with a saturated fat-rich diet. LPS inserts with its lipid A acyl chain into lipoprotein phospholipids. Thereby LPS passes the intestinal barrier by co-migrating with chylomicrons, that are taken up predominantly at the small intestines ileum (Harris et al., 2002). After a fat rich food intake a significant rise of glycosyl-phosphatidyl-inositol (GPI)-anchored AP complexed to lipoproteins is detected in lymph as well (Nauli et al., 2003).

The physiological roles of- and the interpretation of AP serum levels are not clear, but a role in detoxification of LPS has emerged from current research. The co-presence of both AP and LPS in chylomicron rich fractions suggest a role for AP in dephosphorylating the gut derived- LPS already at close vicinity. Detoxification can take place both in the intestinal lumen or en-route to or upon presentation to the liver., specifically in this context to Kuppfer cells and the hepatocytes, which clear the chylomicrons from circulation.

Increased serum AP levels are associated with hepatic damage. Upon an endotoxin insult, circulatory AP is redirected to hepatocytes, thereby reducing

circulating AP levels initially (Bentala et al., 2002) through receptor-mediated uptake (asialo-glycoprotein receptor). Hepatocytes also remove the LPS-loaded chylomicrons (Harris et al., 2002) rapidly from circulation with a half life of 5-10 minutes. LPS is next removed through biliary excretion, thereby preventing Kupffer cells, being a major target for circulating LPS to become activated (Harris, 2002). Bentala et al., 2002, showed that Kupffer cells accumulate AP in LPS-insulted animal models as well. This may imply that under normal conditions Kuppfer cells will not be activated since LPS (lipidA moiety), or its derivative MPLS (MPLA, derivative from Lipid A), is primarily presented to hepatocytes through a lipoprotein receptor and next is removed via biliary secretion. However under conditions with excess LPS, Kupffer cells are activated through a TLR-4 (LPS) receptor.

A wide array of animals have AP and several other entities present to counteract a (bacterial) insult, either local or systemic, induced or available as guard/watchdog function. Amongst others activated neutrophils or macrophages express a wide array of inflammatory mediators destined to neutralise the insult. Moieties like, but not restricted to LPS binding protein (LBP), CD14, Apo-E, VLDL, HDL, albumin, immunoglobulin and AP all have been described to serve this function. When such an insult however is not overcome, e.g. in case of a severe Gram negative or positive insult, resulting inflammatory mediators may initiate a systemic inflammatory response syndrome (SIRS).

It was postulated that AP is consumed as a consequence of its catalytic action towards LPS (Poelstra et al., 1997). This implies that subsequently normal levels are to be restored through a controlled mechanism. In patients suffering from septicaemia, it has been observed that increased serum AP may be preceded by reduced AP serum levels (Manintveld and Poelstra, patent application EP 989626940) and that circulating AP would be cleared from circulation upon LPS interaction (Bentala et al., 2002). The increase in subsequent AP-levels therefore may be a feedback mechanism in response to this AP reduction. A mechanism for such a LPS/AP responsiveness has not been depicted to-date.

In inflammatory processes (temporary) increases are found for serum AP. In the context of this invention such an increase of AP is regarded as a natural response of the innate immune system to an LPS insult to tackle these insults and restore natural balance. Increased AP plasma levels are the result of massive shedding of AP from

hepatocytes in response to the LPS insult. It has been observed that LPS induces Phospholipase-D activity (Locati et al., 2001) which in turn has been reported to act upon GPI anchored proteins, amongst which AP (Deng et al., 1996) and e.g. CD14, thereby effectively shedding the proteins into circulation (Zhang F et al., 2001, Locati M. et al., 2001).

Circulating plasma AP – predominantly anchorless livertype AP (Ahn et al., 2001)- may thus already have exerted its LPS detoxificating activity at the plasma membrane surface and is subsequently freed from the hepatocyte membrane into circulation prior to its subsequent elimination from circulation by e.g. the asialo glycoprotein-route.

AP exerts its catalytic activity towards LPS primarily in the vincinity of a membrane, possibly in so-called lipid rafts (drm or detergent-resistant membrane fraction) where it has been reported to reside. Several publications favor such a catalytic activity of AP at a membrane surface, either presented at the tissue level or released into circulation like with circulating liver plasma membrane fragments (LPMF)(e.g. Deng et al., 1996). The increased AP levels observed in chronically inflamed patients may be caused by the suboptimal detoxification of the gut-derived influx of LPS, which is often enhanced under pathological conditions prior to mobilization of hepatic AP.

The treatment of inflammatory diseases accounts for a substantial percentage of the gross medical cost in developed countries and the incidence of these inflammatory diseases is continuously rising due to key factors like ageing of the population and an increasing number of patients having suppressed immune systems as a consequence of medication and treatment of a wide array of diseases like heart disease, auto-immunity disorders and allergies, organ transplantations, cancer chemo- or radiotherapy and infectious diseases like AIDS. To a certain extent these diseases relate to an influx of bacterial LPS. The influx of LPS is often enhanced by a medical condition of a subject, causing an inflammatory process by a malfunctioning or non-balanced innate immune system, which constitutes the first line of defense against e.g. microbial insults, in particular from LPS / endotoxin producing bacteria.

The current invention is aimed at providing new methods and compositions for the detoxification, neutralisation or complexation of LPS in situ at mucosal tissues in

body cavities before LPS can pass through the mucosal layer and enter the circulation where it would elicit toxic effects and/or an inflammatory response.

Description

Definitions:

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria. Endotoxins are invariably associated with Gram-negative bacteria whether the organisms are pathogens or not. Although the term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin, it is properly reserved to refer to the lipopolysaccharide or LPS complex associated with the outer membrane of Gram-negative bacteria such as *Escherichia (E. coli)*, *Salmonella*, *Shigella*, *Pseudomonas (Ps. aeruginosa)*, *Neisseria (N. meningitidis)*, *Haemophilus (H. influenzae)*, *Chlamydia (Chl. pneumoniae)*, *Helicobacter (H. pylori)* and other leading pathogens.

Lipopolysaccharides are complex amphiphilic molecules with a monomeric molecular weight of about 10 kDa, that vary widely in chemical composition both between and among bacterial species. LPS consists of three components or regions, Lipid A, an R polysaccharide and an O polysaccharide. Lipid A contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. The Core (R) antigen or R polysaccharide is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. Two unusual sugars are usually present, heptose and 2-keto-3-deoxyoctonoic acid (KDO), in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin).

With minor variations, the core polysaccharide and lipid A is common to all members of a bacterial genus (e.g. *Salmonella*), but it is structurally distinct in other genera of Gram-negative bacteria. *Salmonella*, *Shigella* and *Escherichia* have similar but not identical cores.

The biological activity of endotoxin is associated with the lipopolysaccharide (LPS). Toxicity is associated with the lipid component (Lipid A) and immunogenicity is associated with the polysaccharide components. The cell wall antigens (O antigens) of Gram-negative bacteria are components of LPS. LPS elicits a variety of inflammatory responses in an animal. Because it activates complement by the

alternative (properdin) pathway, it is often part of the pathology of Gram-negative bacterial infections.

The Limulus assay (LAL) is a well known bioassay in the art to measure LPS concentrations and toxicity. The assay is based on an exquisitely sensitive primitive defense system of the ancient horseshoe crab, *Limulus polyphemus*. An assay based on this system can be measured by a color change after cleavage of chromogenic or fluorogenic substrates. LAL can used to measure sub-picogram quantities of these microbial products very rapidly with minimal equipment and can detect live, dead and non-cultivable organisms. The blood cells of Limulus, or amebocytes, of the horseshoe crab constitute a primitive "innate" immune defense, binding to the outer cell wall structures of the microbial cell and causing a blood clotting reaction. Soluble LPS, as well as cell wall components of other microbes, such as beta glucans in yeast and fungi, have been shown to cause the horseshoe crab blood to clot. This clotting reaction is now known to be an enzyme cascade whose components are present in granules within the amebocyte. A lysate of the amebocyte is produced by collecting blood cells in a sterile, endotoxin-free method and is available as a commercial product (LAL, Charles River Endosafe, Charleston, S.C.) currently used as an assay for LPS and detoxification of LPS by AP enzymes and compositions comprising sources of AP.

Alkaline phosphatase (AP): EC 3.1.3.1 according to IUBMB Enzyme Nomenclature, the common name is alkaline phosphatase (AP), an enzyme that catalyzes the reaction of a phosphate monoester + H_2O = an alcohol + phosphate. Other name(s) for AP are alkaline phosphomonoesterase; phosphomonoesterase; glycerophosphatase; alkaline phosphohydrolase; alkaline phenyl phosphatase; orthophosphoric-monoester phosphohydrolase (alkaline optimum). The systematic name of AP is phosphate-monoester phosphohydrolase (alkaline optimum).

AP is a wide specificity enzyme, it also catalyses transphosphorylations. In humans and other mammals, at least four distinct but related alkaline phosphatases are known. They are intestinal, placental, placental-like, and liver/bone/kidney (or tissue non-specific) alkaline phosphatase. The first three are located together on chromosome 2 while the tissue non-specific form is located on chromosome 1. The exact physiological functions of the APs are not known, but AP appears to be involved with a large number of physiological processes, among which the detoxification of LPS

through dephosphorylation of the toxicity determining lipid A moiety of LPS. For the current invention, the term alkaline phosphatase may comprise any enzyme exhibiting detoxification of LPS as determined by a Limulus assay or another bioassay. The activity of an AP enzyme or composition or preparation comprising AP can be determined by detoxification of commercially available LPS (for instance Lipopolysaccharide (LPS) from Sigma, Cat. No.L-8274) in vitro, followed by a standard Limulus assay (LAL) before and after AP treatment. Alternatively LPS toxicity reduction through AP activity can be quantitated by means of a bioassay as described by Beumer et al., 2003.

Mucosa is a mucus-secreting membrane lining all body cavities or passages that communicate with the exterior. Mucosa is a moist tissue that lines many organs (such as the intestines) and body cavities (such as nose, mouth, lungs, vagina, bile duct, esophagus) and secretes mucous (a thick fluid). The mucosa, or mucous membrane, is a type of tissue protects body cavities from environmental conditions, pathogens and toxic substances and are usually moist tissues that are bathed by secretions (such as secretions in the bowel, lung, nose, mouth and vagina).

Detailed description of the invention

The current invention is aimed at providing new methods and compositions for the detoxification of LPS in situ at mucosal tissues in body cavities. LPS thus is detoxified before, after or during passage through mucosal layers and entering the circulation where it will exert its toxic effects and/or cause a local or systemic inflammatory response. Detoxification may also comprise neutralising or complexation of LPS by AP, which by close proximity may form a detoxified composition. The methods comprise the use of sources of alkaline phosphatase, which is known to be a potent means for LPS detoxification. A source of AP can be any AP enzyme, or any composition comprising the AP enzyme and any means which is capable of producing a functional AP enzyme in the context of the current invention, such as DNA or RNA nucleic acids encoding an AP enzyme. The nucleic acid encoding AP may be embedded in suitable vectors such as plasmids, phagemids, phages, (retro)viruses, transposons, gene therapy vectors and other vectors capable of inducing or conferring production of AP. Also native or recombinant micro-organisms, such as bacteria, fungi,

protozoa and yeast may be applied as a source of AP in the context of the current invention.

In a first embodiment the invention provides a method for the prevention or reduction of toxic LPS influx through a mucosal lining of a mammalian body cavity comprising the step of administering a source of AP at the mucosal layer. For those jurisdictions where methods of treatment are unpatentable by law, the invention likewise pertains to the use of AP as defined above, or the use of a composition containing a source of alkaline phosphatase as defined above. The source of AP is used for the manufacture of a medicament for delivery of AP at a mucosal layer for the prevention or reduction of toxic LPS influx through a mucosal lining of a mammalian body cavity.

In particular the above mentioned method of administering a source of AP at mucosal layers of body cavities is suited for the treatment or profylaxis of LPS mediated or exacerbated diseases, although the method may also be advantageously used for healthy subjects as a prophylactic treatment aimed at the prevention of LPS induced toxicity and/or LPS induced or exacerbated diseases. The beneficial effects of AP administration to reduce toxic LPS levels in body cavities and at mucosal layers to decrease LPS influx through mucosal layers according to the current invention will generate a general health promoting effect regardless of the medical condition of the subject treated. An LPS mediated or induced disease may be any disease, symptom or group of symptoms caused by LPS toxicity. An LPS exacerbated disease may be any disease or symptom that is not directly caused by LPS or LPS toxicity but a disease which symptoms and clinical features may be aggravated by LPS and the clinical state of the subject suffering from such a disease is worsened by LPS and LPS toxicity.

Preferably the method is aimed at the treatment of an LPS mediated or exacerbated diseases selected from the group consisting of: inflammatory bowel diseases, sepsis/septic shock, systemic inflammatory response syndrome (SIRS), Meningococcemia, trauma/hemorrhagic shock, burn injuries, cardiovascular surgery/ cardiopulmonary bypass, liver surgery/transplant, liver disease, pancreatitis, (necrotising) enterocolitis, periodontal disease, pneumonia, cystic fibrosis, asthma, coronary heart disease, congestive heart failure, renal disease, hemolytic uremic syndrome, kidney dialysis, autoimmune diseases, cancer, Alzheimer, rheumatoid arthritis, lupus, systemic lupus erythematosus.

Circulating endotoxin has been detected in patients with inflammatory bowel diseases, in particular in patients diagnosed with Crohn's disease and ulcerative colitis. Its presence is the consequence of the damaged intestinal mucosa and increased LPS influx or gut translocation and causes or exacerbates the inflammatory response in the intestines. Intestinal bacterial translocation and LPS gut translocation is also observed in acute pancreatitis and liver diseases caused by cirrhosis, alcohol abuse, obstructive jaundice and other hepatic conditions. Endotoxin has also been implicated in the development of periodontal disease, where it penetrates the gingival epithelium / mucosa, ensuing a local inflammatory response. In a preferred embodiment the method comprises oral administration of a source of AP to reduce LPS toxicity at and/or passage of LPS through the mucosa.

The preferred mode of administration comprises the use of pharmaceutical compositions comprising sources of AP, which may be delivered in a daily doses regimen to reduce toxic LPS levels in the lumen of the GI tract for a prolonged period of time. Preferably the pharmaceutical compositions comprise an enteric coating to protect AP from the detrimental effects of gastric juices (pH 1.0 to 2.5) and ensure efficient delivery of AP at the mucosa of the intestinal tract. More preferably, the pharmaceutical composition is a source of AP comprised within an enteric coat.

Enteric coatings arrest the release of the active compound from orally ingestible dosage forms. Depending upon the composition and/or thickness, the enteric coatings are resistant to stomach acid for required periods of time before they begin to disintegrate and permit slow release of AP (drug) in the lower stomach or upper part of the small intestines. Examples of some enteric coatings are disclosed in U.S. Pat. No. 5,225,202 (incorporated by reference). Examples of enteric coatings comprise beeswax and glyceryl monostearate; beeswax, shellac and cellulose, optionally with neutral copolymer of polymethacrylicacid esters; copolymers of methacrylic acid and methacrylic acid methylesters or neutral copolymer of polymethacrylic acid esters containing metallic stearates (for references enteric coatings see: U.S. Pat. Nos. 4,728,512 , 4,794,001 , 3,835,221 , 2,809,918 , 5,225,202 , 5,026,560, 4,524,060, 5,536,507). Most enteric coating polymers begin to become soluble at pH 5.5 and above, with a maximum solubility rates at pH above 6.5. Enteric coatings may also comprise subcoating and outer coating steps, for instance for pharmaceutical compositions intended for specific delivery in the lower GI tract, i.e. in the colon (pH

6.4 to 7.0, ileum pH 6.6), as opposed to a pH in the upper intestines, in the duodenum of the small intestines the pH ranges 7.7-8 (after pancreatic juices and bile addition). The pH differences in the intestines may be exploited to target the enteric-coated AP composition to a specific area in the gut. It also allows the selection of a specific AP enzyme that is most active at a particular pH in the intestine. For instance CIAP (calf intestinal AP) and human placenta (HPLAP) AP are most active at alkaline pH 8.2 in the smallintestinal duodenum, jejunum and ileum, whereas milk derived AP and Bone/Liver/Kidney or Tissue non specific AP (TSN AP) are most active at neutral pH and better suited for treatment of the colon (pH 7.4).

The most preferred mucosal tissues to be treated according to the current invention are the mucosal tissues lining the intestinal tract body cavities. Orally administered AP is delivered at the mucosal tissues of the GI tract, which comprises the esophagus, stomach, the small intestines or bowel, (duodenum, jejunum, ileum) and large intestines or colon (caecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum and anus). Within the scope of the current invention, also mucosal tissues lining the mouth, the ducts of the bile and the pancreas are part of the intestinal tract and may be treated according to method of the current invention.

The compositions comprising a source of AP according to the current invention are particularly suited for oral administration to prevent treat, reduce, treat or alleviate inflammatory diseases of the gastrointestinal tract. Inflammatory diseases of the gastrointestinal tract may be induced and/or exacerbated significantly by the influx of LPS. A reduction in the amount of toxic LPS in the lumen of the intestines by administration of sources of AP will, through detoxification of the lipid A moiety of LPS, result in a corresponding decrease in the systemic influx of toxic LPS in the circulation of a subject. In a most preferred embodiment, the oral administration of sources of AP are particularly preferred for the prophylaxis or treatment of the following inflammatory disease of the gastrointestinal tract: Crohn's disease, colitis, (necrotizing) enterocolitis, colitis ulcerosa, hepatobiliary disease, hepatitis B, hepatitis C, liver cirrhosis, liver fibrosis, bile duct inflammation, biliary obstruction, pancreatitis, acute pancreatitis, peritonitis and periodontal disease.

In another embodiment of the invention, a source of AP is orally administered to subjects who suffer from an increased mucosal permeability of the gastrointestinal tract. Increased mucosal permeability of the GI tract is often the result of a decreased

perfusion or ischemia of the intestines. Ischemia, a lack of oxygen supply by the bloodstream, may be caused by heart failure, injuries, trauma or surgery. Ischemia of the intestines results in a malfunctioning of the mucosa and a consequential increase in the influx or translocation of toxic LPS from the gut, resulting in both local and systemic toxicity and inflammation. The toxicity and inflammatory response may even further enhance the mucosal permeability, resulting in a vicious circle. Increased mucosal permeability of the GI tract may be the result of inflammatory bowel diseases or other pathological conditions of the GI tract. Oral administration of sources of AP according to the current invention will significantly reduce or abolish this increased influx of toxic LPS by detoxification of LPS in the lumen of the intestinal cavities. Exogenous administration of AP will break the vicious circle of LPS influx through the mucosa, inflammation and enhanced permeability of the mucosa resulting in an enhanced LPS influx. Decreased perfusion or ischemia of the intestines and a concomitant increased LPS influx is observed by the following group of diseases or conditions: burns, trauma and/or wounds which may result from accidents, gunshot or knife wounds, surgery, and in particular surgery with cardiopulmonary bypass. Also malfunctioning of the heart function, such as congenital heart disease, congestive heart failure, coronary heart disease and ischemic heart disease may result in ischemia of the intestines and an increased influx of LPS. It is a preferred embodiment of the current invention to treat subjects suffering from this group of diseases and conditions with timely and regular oral administration of compositions comprising a source of AP to prevent or reduce LPS influx through the intestinal mucosa with an enhanced permeability for LPS.

In another embodiment the current invention is aimed at providing a source of AP at the mucosal lining the respiratory tract. The respiratory tract is another body cavity with a mucosal lining that is exposed to the toxic effects of LPS. LPS, either free or associated with inhaled bacteria, enters the respiratory tract bronchial and pulmonary mucosa via normal respiration, by inhalation of e.g. dust-particles, or from infections of the respiratory tract and mucosal tissues with gram negative bacteria. In addition, tobacco is known to be a rich source of LPS and smoking, either passive or active, may further contribute significantly to the LPS burden of the bronchial and pulmonary mucosa. Under normal conditions this LPS is detoxified by the local mucosal immune defence system in the respiratory tract. Therefore, in another preferred embodiment the

current invention pertains to the administration of a source of AP via inhalation to the bronchial and pulmonary mucosa to prevent or reduce LPS influx through the mucosa of the respiratory tract for those conditions where the normal defense responses to LPS are malfunctioning. The current invention also provides compositions suitable for the delivery of AP at the bronchial and pulmonary mucosa. These compositions are preferably administered to for the prophylaxis or treatment of inflammatory diseases of the respiratory tract. In a most preferred embodiment pulmonary administration of a source of AP according to the current invention is applied to treat or prevent a disease selected from the group consisting of pneumonia, lung infections, asthma, CARA, cystic fibrosis, bronchitis and emphysema. The current invention also provides spraying devices, loaded with a composition comprising a source of AP and optionally various excipients such as propellants, carriers, nebulisers and/or diffusers, suitable for the administration of AP at the pulmonary and bronchial mucosa. Spraying devices, inhalators and nebulisers are known in the art of pharmaceutical formulation and will be obvious to the skilled artisan, reference Remmington's Pharmaceutical Sciences, Mace Publishing Company, Philadelphia PA, 17th ed. 1985.

In yet another embodiment, the current invention is aimed at the topical administration of a source of AP at a mucosal layer lining a body cavity. In a preferred embodiment the body cavity is the nasal cavity, oral cavity, vagina or rectum. Topical administration of a source of AP at a mucosal tissue lining a body cavity is preferably applied to treat local or systemic inflammatory diseases, and it is particularly preferred for the treatment or prophylaxis of infections of the nasal, vaginal, oral or rectal cavities, sexually transmitted diseases and infections, urinary tract infections, bladder infections and periodontal disease.

The current invention also provides compositions comprising a source of AP, amongst which are pharmaceutical and nutraceutical compositions comprising a source of AP. The compositions may optionally comprise pharmaceutically acceptable excipients, stabilizers, activators, carriers, permeators, propellants, desinfectants, diluents and preservatives. Suitable excipients are commonly known in the art of pharmaceutical formulation and may be readily found and applied by the skilled artisan, references for instance Remmington's Pharmaceutical Sciences, Mace Publishing Company, Philadelphia PA, 17th ed. 1985. In a preferred embodiment the compositions comprising a source of AP are suitable for oral administration and

comprise an enteric coating to protect the AP from the adverse effects of gastric juices and low pH. Enteric coating and controlled release formulations are well known in the art (references as described above). Enteric coating compositions in the art may comprise of a solution of a water-soluble enteric coating polymer mixed with the active ingredient(s) such as AP and other excipients, which are dispersed in an aqueous solution and which may subsequently be dried and/or pelleted. The enteric coating formed offers resistance to attack of AP by atmospheric moisture and oxygen during storage and by gastric fluids and low pH after ingestion, while being readily broken down under the alkaline conditions which exist in the lower intestinal tract.

AP containing compositions for the delivery of AP at mucosal tissues for detoxification of LPS according to the current invention preferably comprise an eukaryotic AP, more preferably a mammalian AP, which may be of the types tissue non-specific AP, such as liver-bone or kidney type, or tissue specific such as placental AP and intestinal AP. Most preferably the mammalian AP is a human or a bovine AP.

In a preferred embodiment of the current invention the source of AP is AP which is preferably produced or isolated from milk, preferably bovine milk. The milk may be obtained from animals that have been bred or genetically modified to produce elevated levels of AP in their milk as compared to wild-type animals. The preparation of AP enriched fractions from milk is known in the art. For instance the milkfat globule membrane enriched or derived fraction is the preferred AP enriched milk fraction and may be routinely obtained by conventional skimming of raw milk. AP isolated from milk may be formulated in pharmaceutical compositions and in food compositions or in nutraceuticals.

In a preferred embodiment the AP containing composition for oral administration of AP to the mucosa of the gastrointestinal tract according to the current invention is a food product or nutraceutical enriched for AP. In one embodiment the food product may be a plant, fruit or vegetable, optionally genetically modified to contain an enhanced level of AP. In another embodiment the AP containing food product or nutraceutical is a dairy product. In particular preparations and compositions containing non-pasteurised milk or fractions thereof, preferably bovine milk, contain high levels of AP and are particularly suited for oral administration as a source of AP according to the current invention.

The current invention also pertains to a method for the preparation of an AP enriched dairy product, preferably milk, a milk fraction or milk product. The method comprises the fractionation of raw milk, preferably bovine milk, pasteurisation of the fractions not containing or not rich in AP and reformulating said fractions with the unpasteurised, AP rich fractions, to obtain a less perishable and AP enriched dairy product. The non pasteurised AP rich fractions may be sterilised by other means, such as, but not limited to, irradiation with UV-, X- or gamma-rays, filtration, pressure, osmotic pressure, chemicals or antibiotics, ensuring that the AP enzyme remains substantially active and that the milkfraction becomes substantially sterile. This dairy product may be used in compositions or administered directly to subjects suffering from or at risk of developing an LPS mediated or exacerbated disease and/or inflammation. However, the AP enriched dairy product may also be offered to healthy subjects as a pharmaceutical or nutraceutical product for the reduction of toxic LPS in the gastrointestinal tract and for the reduction of LPS influx through the gastrointestinal mucosa.

Examples

Example 1

The current invention, and in particular the effectiveness of AP enzymes, preparations and compositions, and different modes of administration of AP may be tested in various animal models for inflammatory bowel diseases that are known in the art. Animal models mimicking human IBD comprise antigen-induced colitis and colitis induced by microbials; other inducible forms of colitis, chemical (for instance trinitrobenzene sulphonic acid (TNBS) in Montfrans et al., 2002), immunological and physical and genetic colitis models (transgenic and knock-out models, see for instance SCID-mice, Davis et al., 2003, IL-10 KO mice, Rennick et al., 2000, SAMP1/Yit mouse, Kosiewicz, et al., 2001 and Strober et al., 2001); adoptive transfer models and spontaneous colitis models (Kosiewicz, M.M. et al., 2001).

The chemically induced Dextran Sulphate Sodium (DSS) colitis model was originally described by Okayasu et al; Gastroenterology, 1990: 98, 694-702, and is a model for human ulcerative colitis. The model comprises acute and chronic ulcerative colitis in mice caused by administration of 3-10% DSS in their drinking water. The morphological changes and changes in the intestinal microflora are similar to those

seen in clinical cases of ulcerative colitis. The colon damage develops due to a toxic effect of DSS on the epithelial cells and to phagocytosis by lamina propria cells, resulting in production of TNF-alpha and IFN-gamma.

Experimental design for acute DSS colitis:

DSS (MW 40.000 obtained from ICN chemicals) is dissolved in acidified drinking water in a concentration of 5 % (w/v) and given ad libitum to female balb/c mice (Harlan). The solution is refreshed every day. After 7 days of treatment, treated and control mice may be sacrificed and the intestines analysed. The total colon is dissected (from caecum to rectum) and its length is recorded. About half of the colon is frozen in liquid nitrogen and cryo sections are made for morphology (HE staining). Also small parts of the spleen and the liver are snap-frozen in liquid nitrogen for immunohistochemical purposes. A small part of the colon is used to prepare tissue homogenates for cytokine measurements. Small colon strips are cultured in RPMI /. 10% FCS for 24 h in absence or presence of LPS. Cytokine secretion (TNF alpha; IL-1 beta; IFN gamma) in the supernatant is measured using specific ELISA assays. Spleen and mesenteric lymph-nodes are dissected and squeezed to prepare single cell suspensions. 4 Peyer's Patches near the colon are dissected and single cell suspensions are made by use of collagenase. Cells are characterized using flow-cytometric techniques. Spleen cells are cultured for 24 h in RPMI / 10% FCS in absence or presence of LPS or Con A. Cytokine secretion (TNF alpha; IL1 beta; IFN gamma) in the supernatant is measured using specific ELISA assays. Feaces are collected and cultured on McConkey agar plates for enterobacteriaceae contents. For total aerobic bacteria content, feaces are cultured on blood agar plates.

Results

The assays described above are used to determine the effectiveness of compositions comprising AP *in vivo*. Reductions in cytokine secretion are observed; decreases in TNF alpha, Il-1 beta and IFN gamma levels are measured in the inflamed intestines upon oral administration of the alkaline phosphatase rich milkfat globule membrane fraction of bovine milk.

Example 2: Prevention of induction of IBD

IL-10 knockout mice, kept under sterile conditions, do not develop IBD like symptoms. Under non-sterile conditions IL-10 knock out mice develop IBD within

several weeks. Under the latter conditions, daily feeding with AP enriched milk fractions prior to food intake is maintained during 4 week period, and at several time intervals histochemistry and histopathology is performed as in example 1.

Results:

Reduction of histopathology is observed with AP administration and a prevention or delayed onset of IBD is apparent in AP treated mice.

Example 3: Prevention of IBD induction

In the DSS induced colitis model in mice as decribed in example 1, AP is administered daily prior to or after TNBS/DSS induction of IBD. As a result the net effects of DSS/TNBS are reduced and a reduction of histopathology is observed with AP administration and a prevention or delayed onset of IBD is apparent in AP treated mice.

Figures

Figure 1 explains the model of the current invention in three stages, 1. Mucosa in healthy condition, with normal AP histochemistry and LPS detoxification . 2. Diseased condition, deficient AP staining, insufficient detoxification of LPS by AP, influx/ translocation of toxic LPS from the gut into circulation leading to an inflammatory response 3. Restoration of mucosal AP levels and detoxification of LPS by providing an exogenous source of AP.

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Claims

1. The use of a source of alkaline phosphatase for the manufacture of a medicament for the prevention or reduction of (toxic) LPS influx through a mucosal lining of a mammalian body cavity.

2. The use according to claim 1, wherein the prevention or reduction of LPS influx is for the profylaxis or treatment of an LPS mediated or exacerbated disease.

3. The use according to claims 1 or 2, wherein the LPS mediated or exacerbated disease is selected from the group consisting of inflammatory bowel disease, sepsis/septic shock, systemic inflammatory response syndrome (SIRS), Meningococcemia, Trauma/hemorrhagic shock, burn injuries, cardiovascular surgery/cardiopulmonary bypass, liver surgery/transplant, liver disease, pancreatitis, necrotizing enterocolitis, periodontal disease, pneumonia, cystic fibrosis, asthma, coronary heart disease, congestive heart failure, renal disease, hemolytic uremic syndrome, kidney dialysis, autoimmune diseases, cancer, Alzheimer, rheumatoid arthritis, lupus, systemic lupus erythematosus.

4. The use according to any of the preceding claims wherein the source of alkaline phosphatase is administered orally.

5. The use according to any of the preceding claims wherein the body cavity is the gastro-intestinal tract.

6. The use according to any of the preceding claims wherein the source of alkaline phosphatase is administered for the profylaxis or treatment of an inflammatory disease of the GI tract.

7. The use according to any of the preceding claims wherein the inflammatory disease of the gastrointestinal tract is selected from the group consisting of: inflammatory bowel disease, Crohn's disease, colitis, colitis ulcerosa, hepatobiliary disease, hepatitis B, hepatitis C, liver cirrhosis, liver fibrosis, bile duct inflammation, biliary

obstruction, pancreatitis, acute pancreatitis, peritonitis, periodontal disease, enterocolitis, necrotising enterocolitis.

8. The use according to any of the preceding claims wherein the mucosal permeability of the gastro-intestinal tract for LPS is enhanced by a decreased perfusion or ischemia of the intestines.

9. The use according to claim 8 wherein the decreased perfusion or ischemia of the intestines is caused by cardiopulmonary bypass, surgery, trauma/wounds, burns, cardiac surgery, congenital heart disease, congestive heart failure, coronary heart disease, ischemic heart disease.

10. The use according to claim 1 wherein said source of alkaline phosphatase is administered via inhalation.

11. The use according to claim 10 wherein the body cavity is the bronchial and /or the pulmonary mucosa of the respiratory tract.

12. The use according to claims 10 or 11 wherein the composition is administered for the prophylaxis or treatment of an inflammatory disease of the respiratory system.

13. The use according to any one of claims 10 to 12 wherein the disease is selected from the group consisting of pneumonia, lung infections, asthma, cystic fibrosis, bronchitis, emphysema.

14. The use according to claim 1 wherein said source of alkaline phosphatase is administered topically at a mucosal layer.

15. The use according to claim 14 wherein the body cavity is selected from the group consisting of the nasal cavities, oral cavities, vagina and rectum.

16. The use according to claims 14 or 15 wherein the composition is administered for a local or systemic inflammatory disease.

17. The use according to any one of claims 14 or 16 wherein the disease is selected from the group consisting of infections of nasal, vaginal, oral, rectal cavities, vaginitis, sexually transmitted diseases and infection, urinary tract infections, periodontal disease.

18. A composition comprising a source of alkaline phosphatase, optionally comprising pharmaceutically acceptable stabilisers, activators, carriers, permeators, propellants, disinfectants, protectants, diluents, nutrients and other excipients for delivering alkaline phosphatase at the mucosa of a body cavity.

19. The composition according to claim 18 wherein the source of alkaline phosphatase is enterally coated for oral administration and delivery to the intestinal mucosa.

20. The composition of claims 18 or 19 wherein the alkaline phosphatase is a mammalian intestinal alkaline phosphatase, tissue non specific alkaline phosphatase, placental alkaline phosphatase and liver alkaline phosphatase.

21. The composition according to any one of claims 18 to 20 wherein the alkaline phosphatase is human or bovine.

22. The composition according to any one of claims 18 to 21 wherein the composition is an alkaline phosphatase enriched food product or nutraceutical, suitable for oral ingestion and delivery of alkaline phosphatase to the mucosal lining of the gastro-intestinal tract.

23. The composition according to any one of claims 18 to 22 wherein the food product is an, optionally genetically modified plant, vegetable or fruit comprising a source of alkaline phosphatase.

24. The composition according to any one of claims 18 to 23 wherein the food product is a dairy product comprising a source of alkaline phosphatase.

25. The composition according to any one of claims 18 to 24 wherein the dairy product is a non-pasteurised or partially pasteurised milk or milk fraction.

26. The composition according to any one of claims 18 to 25 wherein the milk fraction. is the milkfat globule membrane fraction.

27. Inhaling or spraying device loaded with a composition as defined in any one of claims 18 to 26 and a propellant and/or a nebuliser.

Abstract

The present invention provides a use for alkaline phosphatase for the manufacture of a medicament for the prevention or reduction of toxic LPS influx through a mucosal lining of a mammalian body cavity. A source of alkaline phosphatase is administered for the prophylaxis or treatment of LPS mediated or exacerbated diseases. The invention also provides compositions comprising a source of alkaline phosphatase for the prevention or reduction of (toxic) LPS influx or passage through mucosal layers.